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Homologous recombination promoted by reverse transcriptase during copying of two distinct RNA templates

MATTEO NEGRONI*, MIRIA RICCHETTI*, PASCAL NOUVEL†, AND HENRI BUC*

*Unité de Physicochimie des Macromolécules Biologiques (Unité de Recherche Associée, 1149 du Centre National de la Recherche Scientifique) and †Unité de Génétique des Mammifères, Institut Pasteur, 75724 Paris Cedex 15, France

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ABSTRACT **Retroviruses are known to mutate at high rates. An important source of genetic variability is recombination taking place during reverse transcription of internal regions of the two genomic RNAs. We have designed an *in vitro* model system, involving genetic markers carried on two RNA templates, to allow a search for individual recombination events and to score their frequency of occurrence. We show that Moloney murine leukemia virus reverse transcriptase alone promotes homologous recombination efficiently. While RNA concentration has little effect on recombination frequency, there is a clear correlation between the amount of reverse transcriptase used in the assay and the extent of recombination observed. Under conditions mimicking the *in vivo* situation, a rate compatible with *ex vivo* estimates has been obtained.**

Recombination is one of the most frequent genomic alterations introduced during reverse transcription (1–3). It can allow repair of lethal mutations and is one of the major sources of capture of oncogenes, of rescue of defective retroviruses, and of generation of wider host range viruses (4–7). In mice, recombination between different endogenous retroviruses can lead to the generation of pathogenic retroviruses (8). Recombination seems to occur mainly during RNA-directed synthesis of minus-strand DNA (9–11).

Recombination events taking place during minus-strand synthesis can be divided into two classes, depending on the region of the genome involved. (*i*) Strong-stop recombination results from strand transfer occurring at the ends of the viral genome. The reaction involves the terminal repeated sequence of genomic RNA and is essential for proviral DNA synthesis and generation of the proviral promoter (12, 13). This type of recombination has been studied *in vitro* using model templates and purified reverse transcriptases (RTs) from various sources; a detailed model for this mechanism has been proposed (14–16). (*ii*) The second type of recombination is responsible for reassortment of sequences lying within internal regions of the genome (3, 11, 17, 18). In this case, a copy-choice model, postulating an extensive sequence homology and a sequence-dependent template switch during minus-strand synthesis, has been proposed (9). *In vitro* studies have previously shown a correlation between the presence of a pause site on a given RNA template and the ability for RT to switch synthesis onto a homologous region of a recipient DNA oligonucleotide (19).

We have developed an *in vitro* model system to screen and study homologous recombination events occurring during reverse transcription of internal regions of two RNA templates. We report here the results obtained using Moloney murine leukemia virus (MoMLV) RT.

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MATERIALS AND METHODS

Plasmid Construction. Plasmid pSP6 was obtained by insertion at the *Dra* III site in pBluescript$^-$ (Stratagene) of a single *Sna*BI site followed by the sequence of SP6 RNA polymerase promoter. Deletion of the 106-bp *Sac* I/*Kpn* I fragment resulted in pSP6M. This plasmid, pSP6M, was then modified to give rise to the required DNA sequences. Plasmid pΔLA was obtained by excision of the 445-bp fragment defined by the two *Pvu* II sites present in pSP6M. pLK was constructed by replacing the β-lactamase gene present in pSP6M with the kanamycin-resistance (Kn^r) gene carried in pRSV-neo (20). pΔLA and pLK thus bear an 899-nt common region spanning from the end of the antibiotic-resistance cassette to the first 163 nt of the *lacZ'* gene and containing the origin of replication. ΔP DNA (used for ΔP RNA synthesis) was prepared by linearizing pΔLA at its *Sna*BI site and digesting it further with *Pvu* II. ΔP DNA is the fragment containing the SP6 promoter and going from the unique *Sna*BI site to the unique *Pvu* II site present in pΔLA. All the cloning protocols were performed according to standard procedures (21). Details of the constructions are available on request.

RNA Synthesis. ΔLA (or ΔP) and LK RNAs were independently prepared from pΔLA (or ΔP DNA) and pLK, respectively, by incubation of *Sna*BI linearized plasmid with 40 units of SP6 RNA polymerase for 1 h at 37°C followed by addition of 1 unit of DNase I and further incubation for 10 min at 37°C. RNAs were purified by phenol/chloroform extraction and by two precipitations with 2.5 M ammonium acetate. Resulting RNAs were analyzed by agarose gel electrophoresis and quantified by spectrophotometry.

Primer Sequences. The following sequences were used: STDW, 5'-GACTAGGCTAGCCTATTGGTTAAAAAATGAGCTG-3'; STUP, 5'-CCGCATGCTAGCTAGTACGTGAACCATCACCC-3'; D, 5'-AGATCCTTTTTGATAATCTC-3'; M13, 5'-GTAAAACGACGGCCAGT-3'.

Reverse Transcription. RT concentration was deduced from titration experiments performed with a constant amount of primer/template and increasing amounts of enzyme under initial velocity conditions as described (22). RNA templates were annealed to primer STDW. Subsequently, MoMLV RT (either RNase H$^+$ or RNase H$^-$; both from GIBCO/BRL) was added. The reaction buffer contained 50 mM Tris·HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 1 mM each dNTP, 1 mM dithiothreitol, 100 units of RNasin (Promega). Incubation was carried out for 1 h at 37°C in a final vol of 10 μl. The reaction was stopped by phenol/chloroform extraction followed by incubation for 30 min at 37°C with 1 μg of DNase-free RNase (Boehringer Mannheim) and 0.5 unit of *Escherichia coli* RNase H. DNA was then purified by chromatography on G-50 columns. The presence of full-length DNAs was verified by PCR amplification performed in 10 mM Tris·HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM each dNTP, 1 μM each

Abbreviations: RT, reverse transcriptase; MoMLV, Moloney murine leukemia virus; Kn, kanamycin; Ap, ampicillin.

primer (STDW and STUP), and 2.5 units of *Taq* DNA polymerase (Roche–Cetus, Alameda, CA). PCR products were analyzed by agarose gel electrophoresis.

Cloning of Reverse Transcription Products. Second-strand synthesis was achieved by using the STUP primer and the same conditions as in the PCR but heating the samples at 94°C for 90 sec in the absence of *Taq* polymerase, which was then added at 55°C for 1 min. Incubation was subsequently carried out at 72°C for 10 min and the reaction was stopped by phenol/chloroform extraction. Full-length double-stranded DNAs were gel purified. The purification was carried out by removing the gel slice spanning from the LK to the ΔLA DNA bands, allowing the recovery of both parental and recombinant DNAs. Eluted DNA was incubated with 0.2 unit of the Klenow fragment of *E. coli* DNA polymerase I in the absence of nucleotides at 25°C for 10 min and, after the addition of 50 μM each dNTP, for a further 60 min. The reaction was stopped by phenol/chloroform extraction followed by chromatography on G-50 columns. DNA was then digested with *Nhe* I, circularized by 0.32 unit of T4 DNA ligase, and used for transformation of XL1-Blue MRF′ Epicurian Coli cells (Stratagene).

Statistical Analysis. Statistical analysis was carried out by computing the value of p as follows:

$$p = \left| \frac{f_1 - f_2}{\sqrt{q(1-q)\left(\frac{1}{wt_1} + \frac{1}{wt_2}\right)}} \right|,$$

where, if r_1 and r_2 are the number of recombinant clones in the two samples and wt_1 and wt_2 are the number of parental clones, $q = (r_1 + r_2)/(wt_1 + wt_2)$, $f_1 = r_1/wt_1$, and $f_2 = r_2/wt_2$. Resulting p values were compared to the table of T distribution of Student's law (23).

RESULTS

The Recombination Assay. We have designed an unbiased *in vitro* model system for RT-mediated RNA recombination where we have tried to mimic the *in vivo* situation: use of two long RNA molecules, 1:1 ratio of donor to acceptor RNA, RT concentration $\geq 10^{-7}$ M. The two templates are 2500 (ΔLA) and 3200 (LK) nt (Fig. 1; see *Materials and Methods*). Each one carries, in a common central portion, the sequence of a plasmid origin of replication (pBluescript), flanked by one antibiotic-resistance gene [ampicillin (Ap) and Kn for ΔLA and LK, respectively] and the *lacZ′* gene (functional in LK and partially deleted in ΔLA).

The two RNAs are reverse transcribed in the same test tube. If the RT promotes one strand transfer within the homologous region, two new types of single-stranded DNA will be generated besides the parental ones. After removal of RT, the second DNA strand is synthesized by *Taq* DNA polymerase. These DNAs are plasmids, since they carry an origin of replication and an antibiotic-resistance gene, and once circularized by DNA ligase they can be used to transform bacterial cells. Selection on antibiotic-containing plates (either Kn or Ap) allows detection of colonies derived from transformation with recombinant molecules as Ap^r/lac^+ or Kn^r/lac^- colonies (Fig. 1). However, a mutation introduced during copying of the *lacZ′* gene can give rise to artefactual recombinant-like clones on Kn plates. On Ap plates, false recombinants can be due only to cotransformation of a bacterial cell by both parental plasmids. Our analysis was based on the Ap selection system. To overcome the problem of false recombinant colonies, all Ap^r/lac^+ clones were replicated onto Kn-containing plates and Kn^r clones were rejected.

To estimate and to characterize the artefactual recombinant molecules throughout the experimental procedure, a control sample (corresponding to C1 in Fig. 2) was run in parallel to the main experiment (recombination sample R). In these controls, reverse transcription was performed separately for the two types of RNA and the samples were pooled immediately afterward and processed as described above.‡§¶

Evidence for a Copy-Choice Model. The present system allows analysis of any individual DNA molecule produced during reverse transcription. It is then possible to distinguish molecules having the molecular structure expected for recombinants from any artefact. The primers used to initiate DNA synthesis were specifically designed to facilitate such an analysis. The primer used for first-strand synthesis hybridizes internally to both RNA sequences, yielding final products with a characteristic size (Figs. 1 and 3). Moreover, DNA synthesis from these primers suppresses a unique *Sna*BI site present on the plasmids used for RNA production (pLK and pΔLA) and creates a single *Nhe* I site (Fig. 3). For each experiment 2×10^4–10^6 total clones can be obtained, allowing detection of rare events. We systematically analyzed the minimal amount of clones required for a significant statistical analysis. Indeed, numerous $Ap^r/Kn^s/lac^+$ colonies (Kn^s, Kn sensitive) were found among Ap^r/lac^- clones, and all the corresponding plasmids were analyzed by *Nhe* I restriction.

All plasmids reisolated from $Ap^r/Kn^s/lac^+$ clones as well as plasmids from several Ap^r/lac^- clones were analyzed by *Nhe* I restriction. All the plasmids arising from Ap^r/lac^- clones contained a single *Nhe* I restriction site and showed the size expected for plasmids derived from reverse transcription. The same was true for 81% (456/561) and 68% (38/56) of $Ap^r/Kn^s/lac^+$ clones in recombination and control samples, respectively.

To obtain molecular evidence for physical association of the two markers, a PCR test was then performed on this selected *Nhe* I^+ population (Fig. 3). The choice of primers allowed amplification only if the Ap and the undeleted *lacZ′* genes were on the same DNA molecule. Moreover, since the amplification product corresponds to the region of homology between the two RNAs, this assay allows analysis of the 900-nt region where the strand transfer should have occurred. Among the *Nhe* I^+ plasmids, the correct size of the region of homology was also found after PCR analysis (Fig. 3 *Middle*) for 116/116 and 19/19 in R and C1 samples, respectively. Further digestion

‡To understand the origin of recombinant molecules in the control sample three additional controls, C2–C4, were included, mixing LK and ΔLA samples at different steps (see Fig. 2). Obtained recombination frequencies were not significantly different, suggesting that the small number of background clones originate from a common artefact taking place after the C4 step (data not shown). No background signal was detected when 0.6 pmol of cesium-purified supercoiled pΔLA and pLK were used to cotransform the same bacterial strain. Even when purified linear plasmids were recircularized by DNA ligase and mixed just before transformation, the level of intrabacterial recombination was still $<10^{-5}$. The significant level of the background likely results from a large amount of unligated double-stranded DNA.

§To assess the relative efficiency of LK RNA and ΔL RNA as templates for a full reverse transcription, an aliquot from each sample was plated onto Kn-containing plates. The efficiency of reverse transcription of LK RNA was half that observed for ΔL RNA, a result consistent with the larger size of LK RNA. The relative efficiency of the overall reaction was also calculated: 1 pmol of RNA should give 2 pmol, or 3.4 μg, of double-stranded DNA for efficiency of the reaction of 100%. Since the competent cells used give a transformation efficiency of 5×10^8 per μg of cesium-purified supercoiled DNA, 1.7×10^9 clones would be expected. The transformation efficiency using a nonsupercoiled plasmid is 10-fold less. Our experiments give from 2×10^4 to 1×10^6 total clones per 50–100 pmol of starting RNA. Therefore, 1 to 60 RNA molecules out of 10^4 present in the reverse transcription mixture are converted into clonable plasmids.

¶Since *a priori* lac^+ to lac^- reversions due to errors in the *lacZ* gene could significantly affect the pool of Ap^r/lac^+ clones, an aliquot of C1 samples was plated onto Kn-containing Petri dishes; 61 of 5900 Kn^r clones were lac^-. Thus, only one Ap^r/lac^+ clone of 100 is lost, not significantly affecting the recombination frequency deduced from this assay.

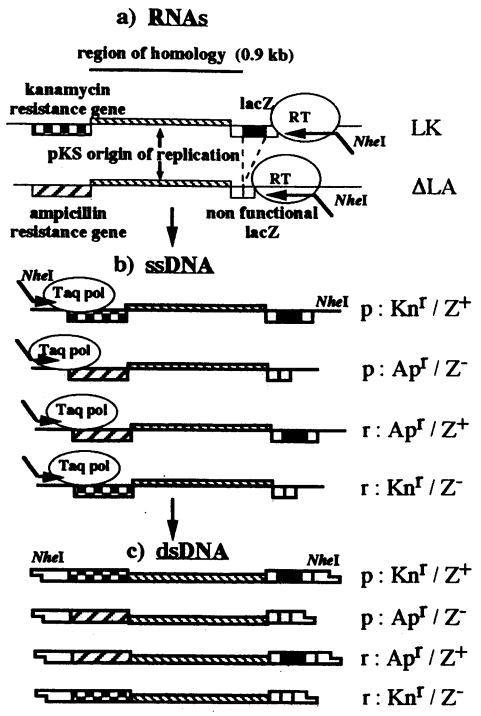

FIG. 1. Basic experimental approach. (*a*) RNA templates LK and ΔLA are schematically represented. Reverse transcription was primed in the same test tube on LK and ΔLA RNAs by using a common STDW primer carrying in its nonhybridizing tail a sequence cleaved by the restriction endonuclease *Nhe* I. (*b*) If homologous recombination occurs during DNA synthesis, two types of single-stranded DNAs will be generated besides the parental ones (shown as p for parental and r for recombinant). They can be used as templates for second-strand synthesis, which is achieved by using *Taq* DNA polymerase. This second polymerization step is initiated after annealing by another common overhanging primer, STUP, also carrying one *Nhe* I site. (*c*) Structure of the four types of double-stranded DNAs expected to be produced in the reaction after *Nhe* I digestion. After ligation and transformation of bacteria, each of these circular DNAs is expected to yield colonies exhibiting the four indicated phenotypes.

by *Hae* II of 26 PCR products from R samples (Fig. 3 *Bottom*) showed no variability in the size of this region (accuracy, ±30 bp). We thus conclude that strand transfer has occurred and is strictly dependent on the presence of a region of homology between the two templates, reproducing *in vitro* the copy-choice model of retroviral recombination (see also ref. 24).

Estimates of Homologous Recombination Rates. Recombinant $Ap^r/Kn^s/lac^+$ clones, consistent with a copy-choice model (presence of one *Nhe* I site, expected plasmid size, occurrence of an amplification product of 1188 bp), were retained for computation of recombination frequencies. When reverse transcription of the two types of RNA was performed in the same test tube under the conditions described in Table 1 (row 1), the frequency of recombinant clones in two independent experiments was 1.1×10^{-3} and 1.2×10^{-3} (rows 1 and 2). These values are significantly higher than those obtained in the corresponding control samples, 2.4×10^{-4} and 2.7×10^{-4}. Therefore, RT is perfectly able to promote reassortment of internal markers even in the absence of other viral components.

Effect of RNA and RT Concentrations on Recombination. A series of experiments was then performed to optimize the strand transfer process. A constant concentration of glycerol was used in all our experiments, and the frequency was measured by using a fixed concentration of RNA template (50 nM each) and variable amounts of RT. For each point, the number of total Ap^r colonies screened was always $>1.4 \times 10^4$. After background subtraction, recombination frequencies

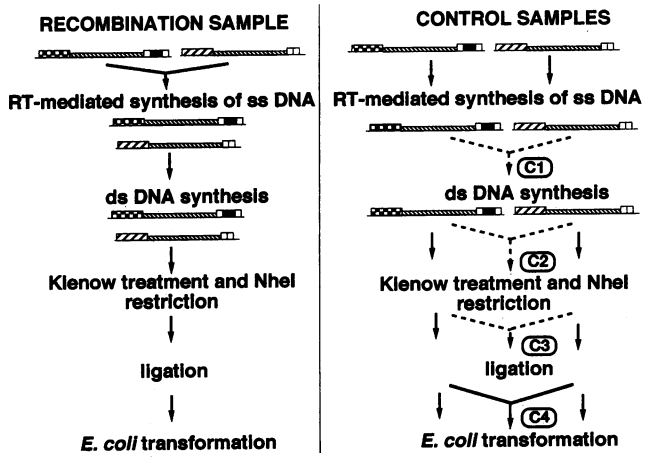

FIG. 2. Scheme of experimental procedure. The two RNAs were mixed either before (recombination sample; *Left*) or after (control sample C1; *Right*) reverse transcription. Symbols are the same as in Fig. 1. C2, C3, and C4 correspond to other controls where reverse transcription products from LK and ΔLA were processed separately and mixed at the various steps indicated. (See footnotes ‡, §, and ¶.) ss, Single stranded; ds, double stranded.

were calculated. As shown in Fig. 4, an increase in recombination frequency was observed when RT concentration was varied from 100 to 500 nM. When the experiment was repeated with twice the RNA concentration (100 nM each), a similar profile was obtained.

These observations suggest that a weak association of the enzyme with the template at a crucial point of the process limits the efficiency of strand transfer. It is also evident that a high concentration of glycerol does favor recombination. Indeed, the frequency increased by a factor 4 when the glycerol concentration was increased from 5% to 7.5% (see Table 1, rows 1–3 and legend).

The maximal frequency obtained with a RNase H^+ MoMLV RT was therefore of the order of 9×10^{-3} (Fig. 4). From these

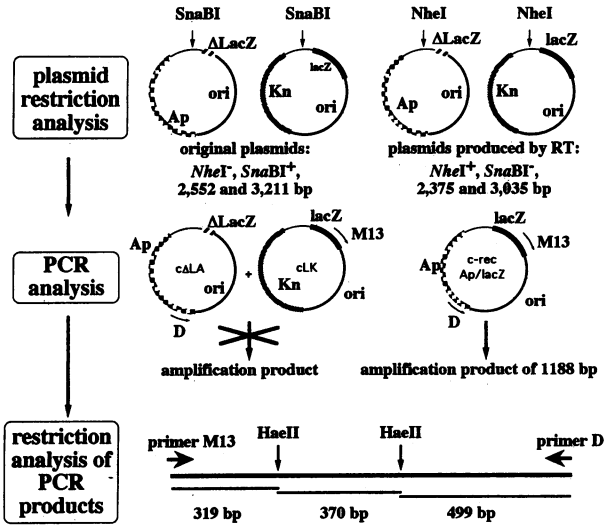

FIG. 3. Analysis of recombinant clones. To identify molecules originating from homologous recombination, plasmids were purified from all the $Ap^r/Kn^s/lac^+$ clones. Each sample was analyzed by *Nhe* I digestion and, in some cases, also by *Pvu* II and *Rsa* I digestion (*Top*). (*Middle*) *Nhe* I^+ plasmids were further analyzed by PCR amplification with primers D and M13 (see *Materials and Methods*). When pΔLA and pLK plasmids were coamplified, no PCR product could be detected. (*Bottom*) Cutting the PCR products with *Hae* II yields a specific pattern.

Table 1. Main parameters affecting recombination frequency

Total RNA concentration, nM	RT concentration, nM	RNAs available for RT	Frequency in recombination sample	Frequency in control sample	Observed recombination frequency	Recombination rate/nt/run of reverse transcription	*P*
200	250	LK and ΔLA	1.3×10^{-3} (45/33,700)	2.1×10^{-4} (3/14,550)	1.1×10^{-3}	2.4×10^{-6}	<0.001
200	250	LK and ΔLA	1.6×10^{-3} (27/16,700)	3.7×10^{-4} (4/10,700)	1.2×10^{-3}	2.7×10^{-6}	<0.001
200	250 (G)	LK and ΔLA	4.9×10^{-3} (98/19,900)	7.1×10^{-4} (13/18,200)	4.2×10^{-3}	9.3×10^{-6}	<0.001
600	250 (RNase H^-)	LK and ΔLA	7.7×10^{-5} (4/51,600)	4.5×10^{-5} (2/44,700)	—	—	<0.6
600	250 (RNase H^+)	LK and ΔLA	1.7×10^{-3} (56/32,700)	3.3×10^{-4} (16/48,900)	1.4×10^{-3}	3.1×10^{-6}	<0.001
600 (ds)	500	LK	$<5.9 \times 10^{-6}$ (0/17,000)	$<7.2 \times 10^{-5}$ (0/13,900)	—	—	—
170	425	LK	1.2×10^{-2} (264/22,700)	1.4×10^{-4} (3/20,700)	1.2×10^{-2}	1.3×10^{-5}	<0.001
200	500	LK	1.6×10^{-2} (85/5288)	3.6×10^{-4} (2/5544)	1.6×10^{-2}	1.8×10^{-5}	<0.001
300	500	LK	1.4×10^{-2} (271/19,000)	2×10^{-4} (16/78,300)	1.4×10^{-2}	1.6×10^{-5}	<0.001

Columns: 1 and 2, RNA and RT concentrations used in each assay; 3, RNA primed for reverse transcription; 4 and 5, recombination frequencies observed in recombination and control samples (number of recombinant colonies among the total colonies is also given); 6, average frequency of recombination obtained after subtraction of background values; 7, recombination rate per reverse transcription, computed as explained in the text; 8, *P* values resulting from statistical analysis carried out as described. Rows: 1 and 2, two independent experiments performed under similar conditions of competitive reverse transcription; 3, effect of glycerol (G) on recombination (in this experiment, the glycerol concentration was 7.5% instead of the 5% usually used); 4 and 5, effect of an RNase H mutation; 6, use of a double-stranded (ds) acceptor molecule; 7–9, reverse transcription of a single template [in this case recombinants were scored as ($Ap^r lac^+$)/($Kn^r lac^+$ + $Ap^r lac^+$) and a correction factor was applied to take into account the different efficiency of the antibiotic selection].

values, an average recombination rate per nucleotide can be computed. On the RNA templates, the region of homology encompasses 900 bases. Each template is used only once and one can score only recombination events occurring between RNA molecules of different types, leading to a new phenotype. As 50% of each template is present in the reaction, the maximal recombination rate obtained when both RNAs are concomitantly reverse transcribed is therefore equal to $2(9 \times 10^{-3})/900$ or 2×10^{-5}.

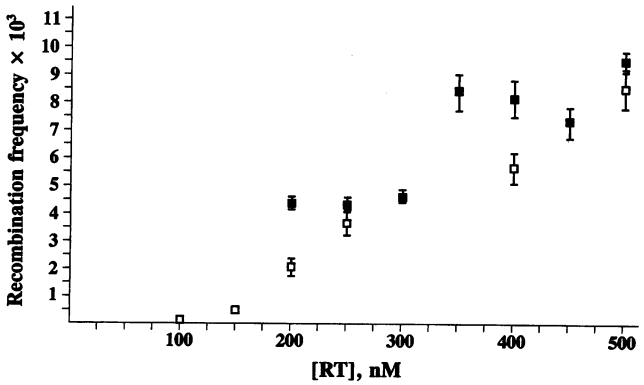

FIG. 4. Recombination frequency as a function of RT concentration. □, 50 nM each RNA; ■, 100 nM each RNA. RT concentration corresponds to the total number of active sites. Standard deviations were computed as $f/\sqrt{n}$, where n is the number of recombinant clones screened and f is the frequency of recombination. In both experiments, the lowest RT concentration tested was the point where RNA and RT were equimolar. The value corresponding to the highest RT concentration in the 200 nM RNA experiment results from two separate experiments, yielding values equal to 8.4×10^{-3} (for a total of 60,150 clones) and 1.0×10^{-2} (26,900 clones).

Effect of the Structure of the Acceptor and Donor Templates. We first checked that when the donor template was not degraded, recombination was inefficient. When a RNase H^- RT was substituted for the wild-type enzyme, the recombination frequency dropped from 1.4×10^{-3} to background level (Table 1, rows 4 and 5). Second, we prepared as a potential acceptor molecule a double-stranded RNA·DNA hybrid obtained by reverse transcription of a ΔLA RNA with an RNase H^- RT. No recombinant clones were detected (row 6).

Finally, the acceptor RNA was kept as a single-stranded molecule (asymmetric reverse transcription). ΔLA RNA was replaced by a RNA having the same sequence but lacking the 3′ region complementary to the primer used to initiate reverse transcription (ΔP RNA; see *Materials and Methods*). We first verified that reverse transcription of ΔP RNA was negligible. The assay was then performed as described above, mixing ΔP RNA with an equivalent amount of LK RNA. Under these conditions, the recombination frequency was maximal and reached a value of $1.4 \pm 0.2 \times 10^{-2}$ (Table 1, rows 7–9). This represents twice the maximal frequency obtained under the conditions where both RNA species were reverse transcribed (see Fig. 4). Such a result is expected if, in the case of competitive reverse transcription, recombination could occur only when the region of homology present on the acceptor template was not yet reverse transcribed at the time of strand transfer.

DISCUSSION

The goal of the study presented here was to investigate *in vitro* homologous recombination occurring during reverse transcription of internal regions of RNA templates, a type of recombination that is an important source of genetic variability in retroviruses. The system was designed so that RT-promoted

recombination could occur only during RNA-directed synthesis of the first DNA strand. Analysis of the resulting plasmids having the expected genotype excluded spurious contaminants.

MoMLV RT can indeed promote recombination between two RNA templates. This occurs via a mechanism requiring sequence homology and in the absence of termination sites for the replication process. Moreover, RNAs devoid of any retroviral sequence were used here. Viral cis-acting elements are therefore not essential for this assay to work. The inability of a RNase H deletion mutant to recombine indicates that, as for strong-stop recombination, the degradation of the template RNA during DNA synthesis is a prerequisite for the strand transfer to occur. Furthermore, strand transfer was dramatically inhibited when the acceptor RNA was complexed to its complementary DNA showing that, at least in the absence of additional proteins, no strand displacement can take place. Hence, RNA regions involved in double-stranded interactions do not constitute an available substrate for a strand-transfer reaction. In this system, nonhomologous recombination was not detected. This could be due either to the low occurrence of these events or to the fact that our experimental system was not optimal to detect this kind of recombination.

The data reported here clearly indicate that homologous recombination can be promoted by RT alone. It is likely that such is also the case for strong-stop recombination where conflicting results have been presented (14–16, 19, 24, 25). These discrepancies can now be easily explained if this process also requires rather high RT concentrations. The template and enzyme concentrations used in the present assay (0.1–0.5 μM) are still largely inferior to the ones prevailing in a virion. The excess of enzyme over template used here (1- to 5-fold) is lower than that existing during *in vivo* reverse transcription (a factor of 10–20).

This is not to deny that, within the virions, other factors can affect the recombination process. First, the present study was performed on long RNA templates that are most likely rich in secondary structures. Any factor, such as the nucleocapsid protein, interfering with the equilibrium between single- and double-stranded forms of nucleic acids (and this is true for the template RNA as well as for the newly synthesized DNA) might play an important role (refs. 26 and 27 and references therein). Second, in our *in vitro* experiments, glycerol markedly increased the recombination frequency, in particular at low enzyme concentrations. Glycerol acts like a nonspecific condensing agent. Again, it could partially mimic the role of a viral protein.

Finally, it is not known whether one or two copies of RNA are reverse transcribed in the virion. Moreover, even if reverse transcription is initiated on both RNA copies, breakage of RNA occurring on one template can lead to premature termination. In this respect, it is reassuring that the maximal recombination frequency was increased by only a factor of 2 when only one template was reverse transcribed. The recombination rate per reverse transcription was therefore maintained within the range of values measured *ex vivo* after a single cycle of replication using a spleen necrosis virus-based vector (2.5×10^{-5}) (3). Refinements introduced in the present model system are not likely to affect the extent of recombination but will probably shed light on the sequence specificity and the detailed mechanism of the strand-transfer process.

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